Did we miss something? Acanthocephalan infection patterns in amphipods: a reappraisal in the light of recently discovered host cryptic diversity

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Acanthocephalan infection patterns in amphipods: a reappraisal in the light of recently discovered host cryptic diversity

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ABSTRACT: Amphipods are model species in studies of pervasive biological patterns such as sexual selection, size assortative pairing and parasite infection patterns. Cryptic diversity (i.e. morphologically identical but genetically divergent lineages) has recently been detected in several species. Potential effects of such hidden diversity on biological patterns remain unclear, but potentially significant, and beg the question of whether we have missed part of the picture by involuntarily overlooking the occurrence and effects of cryptic diversity on biological patterns documented by previous studies. Here we tested for potential effects of cryptic diversity on parasite infection patterns in amphipod populations and discuss the implications of our results in the context of previously documented host–parasite infection patterns, especially amphipod–acanthocephalan associations. We assessed infection levels (prevalence and abundance) of 3 acanthocephalan species (*Pomphorhynchus laevis*, *P. tereticollis* and *Polymorphus minutus*) among cryptic lineages of the *Gammarus pulex*/*G. fossarum* species complex and *G. roeseli* from sampling sites where they occur in sympatry. We also evaluated potential differences in parasite-induced mortality among host molecular operational taxonomic units (MOTUs)–parasite species combinations. Acanthocephalan prevalence, abundance and parasite-induced mortality varied widely among cryptic MOTUs and parasite species; infection patterns were more variable among MOTUs than sampling sites. Overall, cryptic diversity in amphipods strongly influenced apparent infection levels and parasite-induced mortality. Future research on species with cryptic diversity should account for potential effects on documented biological patterns. Results from previous studies may also need to be reassessed in light of cryptic diversity and its pervasive effects.

KEY WORDS: Cryptic diversity · Infection patterns · Parasite-induced mortality · *Pomphorhynchus laevis* · *Pomphorhynchus tereticollis* · *Polymorphus minutus* · *Gammarus pulex*/*Gammarus fossarum* complex · *Gammarus roeseli*

1. INTRODUCTION

Amphipods are a highly speciose crustacean taxon in which cryptic diversity has recently been detected (i.e. morphological undistinguishable but genetically divergent lineages; Hogg et al. 1998, Witt & Hebert 2000, Lefébure et al. 2006, Witt et al. 2006, Sutherland et al. 2010, Lagrue et al. 2014). Cryptic diversity is regularly documented in freshwater amphipods and seems to be widespread geographically and across taxonomic species (Gervasio et al. 2004, Hogg et al. 2006, Bradford et al. 2010, Baird et al. 2011). In Europe, *Gammarus pulex* and *G. fossarum* are widely distributed 'taxonomic' species (Karaman & Pinkster 1977). Although these 2 species are conventionally accepted, their discrimination by morphological

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traits has always been problematic (Müller 1998, Lagrue et al. 2014). Furthermore, substantial genetic divergence exists among geographically isolated populations of both species (Meyran et al. 1997, Müller 1998, 2000, Westram et al. 2011a, Weiss et al. 2014). Recently, multiple cryptic lineages or molecular operational taxonomic units (MOTUs; Floyd et al. 2002, Blaxter et al. 2005) have been detected in eastern France: 8 MOTUs in *G. fossarum* and 4 in *G. pulex*, all morphologically undistinguishable (Lagrue et al. 2014). Genetic determination based on mitochondrial DNA (cytochrome *c* oxidase subunit I sequences) revealed large genetic divergences among MOTUs (up to 27%). Data indicated complex phylogeographical histories in both *G. pulex* and *G. fossarum*, involving several events of geographical isolation allowing allopatric divergence, followed, for some MOTUs, by geographical expansion. MOTUs then experienced multiple and repeated secondary contacts, leading to a complex, mosaic-like, geographical distribution (Lagrue et al. 2014). Similar patterns have been documented in *G. fossarum* in Germany (Weiss & Leese 2016). Interestingly, MOTUs are commonly observed in sympatry with 2 or 3 divergent lineages often co-occurring in amphipod populations. Overall, while *G. pulex* and *G. fossarum* have long been considered as single, but independent, functional taxonomic units (Karaman & Pinkster 1977, Müller 1998), recent evidence shows that the situation is much more complex.

Cryptic diversity also has potentially profound implications for evolutionary biology, biogeography, ecology and conservation (Molbo et al. 2003, Bickford et al. 2007, Cothran et al. 2013a). The existence of sympatric, morphologically cryptic amphipod MOTUs may indeed have significant implications on well-studied and widely accepted ecological patterns (Sutherland et al. 2010, Cothran et al. 2013a,b, Galipaud et al. 2017). This is especially true given that *G. pulex* has been a reference study model in a diversity of research areas such as mate choice, size-assortative mating patterns and host–parasite interactions (Birkhead & Clarkson 1980, Elwood et al. 1987, Bakker et al. 1997). Many studies using *G. pulex* have been conducted on amphipod populations that could or have already proven, in hindsight, to be made of several sympatric cryptic MOTUs of *G. pulex*/*G. fossarum* (Bauer et al. 2000, 2005, Lagrue et al. 2007, 2014). In that regard, previous studies conducted on *G. pulex* may need to be reappraised in light of recent cryptic diversity found in these taxa.

Amphipods are hosts to a suite of helminth parasites, especially acanthocephalans (Bauer et al. 2000, 2005, Westram et al. 2011b). These parasites are also known for their ability to manipulate amphipod hosts in ways that make them more vulnerable to predation by the appropriate definitive hosts (Moore 2002). Some of the most studied parasite models are *Pomphorhynchus laevis*, *P. tereticollis* and *Polymorphus minutus* in their amphipod hosts, *G. pulex* and *G. fossarum*. All 3 species affect amphipod behaviour, phenotype and physiology (Bauer et al. 2000, 2005, Perrot-Minnot 2004, Tain et al. 2006, Kaldonski et al. 2007). As a result, acanthocephalan-infected hosts often experience higher mortality linked to increased predation (Thomas et al. 1995, Rousset et al. 1996, Parker et al. 2003, Benesh & Valtonen 2007). However, cryptic amphipod lineages may differ in their physiology, behaviour and/or ecology and thus in their vulnerability to infection, host manipulation and mortality due to acanthocephalan infection (Cothran et al. 2013a,b, Galipaud et al. 2017). The presence of multiple amphipod MOTUs occurring in sympatry but at varying frequencies could influence the evolution of host–parasite interactions and infection patterns (Lively & Dybdahl 2000, Gandon 2004, Rigaud et al. 2010, Westram et al. 2011b). Generally, among host MOTUs, differences in vulnerability to infection and parasite-induced mortality may confound our perception of such patterns if we do not account for cryptic diversity; for example, contrary to expectations, many *G. fossarum*/*G. pulex* MOTUs showed similar or lower susceptibility to parasite infection and parasite-induced mortality compared to the invasive *G. roeseli* (Galipaud et al. 2017). Amphipod MOTUs also vary in their susceptibility to parasite-induced mortality. As a result, parasite abundance increases with host body size in some MOTUs and decreases in others (Galipaud et al. 2017). Because native MOTUs also vary in mean body size, even when occurring in sympatry, unawareness about cryptic diversity might have biased previous estimations of parasite-induced pathological effects (see also Galipaud et al. 2015a).

Generally, the discovery of cryptic diversity calls for a critical reappraisal of previous findings made at the 'taxonomic' species level, especially in areas where cryptic diversity has now been documented. Misinterpretations of the causes and consequences of several broadly recognized and accepted biological and ecological patterns such as size-assortative pairing, acanthocephalan infection patterns and parasite-induced mortality may exist in the literature. It also begs the question of whether we have missed part of the big picture by involuntarily overlooking the occurrence and potential effects of cryptic di-

versity on the pervasive and widely accepted patterns mentioned above. In rivers sampled in eastern France, 2 out of 3 amphipod populations contained multiple sympatric MOTUs of *G. fossarum* or *G. pulex* (Lagrue et al. 2014). Thus, previous studies documenting infection patterns (e.g. prevalence, abundance and parasite-induced mortality) of amphipods in the same region may have unwittingly missed a potentially significant factor influencing these patterns (Bauer et al. 2000, Bollache et al. 2002, 2006, Rigaud & Moret 2003, Lagrue et al. 2007, 2011, Tain et al. 2007, Kaldonski et al. 2008a). Because previous studies may have missed the importance of cryptic diversity, we used data collected on infection patterns in 5 MOTUs across 9 populations to test for potential implications of cryptic diversity on acanthocephalan infection patterns. We then discuss its significance for interpretations of data published by ourselves and colleagues at the University of Bourgogne (Dijon, France). The present study focusses on cryptic diversity in amphipod hosts, as we assume that acanthocephalan parasites are genetically homogeneous at the scale of our sampling area (Perrot-Minnot et al. 2018, R. Wattier & M. J. Perrot-Minnot unpubl. data). However, we do discuss the potential occurrence and implications of cryptic diversity in parasites on observed host–parasite dynamics in light of other very recent findings (Zittel et al. 2018).

Here, we assessed potential differences in parasite prevalence (proportion of infected hosts in a population, in %), abundance (mean number of parasites per individual host) and parasite-induced mortality in 3 acanthocephalan species separately (*P. tereticollis*, *P. laevis* and *P. minutus*) among cryptic MOTUs of the *G. pulex*/*G. fossarum* species complex (Lagrue et al. 2014). We focussed on sampling sites where at least 2 of these cryptic MOTUs occur in sympatry, including 2 that have been heavily used in previous studies conducted at the University of Bourgogne. We assessed the respective effects of MOTU identity and sampling site (i.e. river) on the infection pattern (prevalence and abundance) and parasite-induced mortality among and within MOTUs, for each parasite species separately. In sampling sites where it occurred, we also assessed infection levels (parasite prevalence and abundance) and mortality in the invading *G. roeseli* for comparison purposes. We tested whether cryptic diversity in amphipod hosts and MOTU identity explain a larger part of observed variance in infection patterns than can be explained by amphipod sampling site. That is, prevalence and abundance, as well as pathological effects of infections (parasite-induced mortality) may vary more among MOTUs than among populations. We then discuss the potential influence of inter-MOTU differences in acanthocephalan infection patterns on the results of previous studies, including some of our own, and the universal significance of cryptic host diversity in studies of amphipods in general and their parasites in particular.

2. MATERIALS AND METHODS

2.1. Field sampling and laboratory processing

Amphipods of the *Gammarus fossarum*/*G. pulex* (hereafter referred to as '*Gf*' and '*Gp*', respectively) species complex were collected using kick nets from different rivers in 2010. Here, we used data from a subsample of 9 sampling sites, including some that have been extensively used in previous studies on mate choice, size-assortative pairing and, most importantly, acanthocephalan infection patterns, host manipulation or parasite-induced mortality prior to the discovery of cryptic diversity. In France, the invasive *G. roeseli* (hereafter '*Gr*') often co-occurs and harbours the same acanthocephalan parasites (Bauer et al. 2000, 2005, Bollache et al. 2006, Westram et al. 2011a). When present, *Gr* was also sampled. Between 365 and 845 amphipods were haphazardly selected and preserved individually in 100% ethanol (Table 1). In the laboratory, samples

Table 1. Distribution (sample size) of *Gammarus fossarum*/*G. pulex* (*Gf*/*Gp*) molecular operational taxonomic units (MOTUs) and *G. roeselii* (*Gr*) among and within sampling sites (river names and GPS coordinates). (–) rivers where MOTUs were absent

Sampling site		MOTU				
River	GPS coordinates	*Gf*-I	*Gf*-III	*Gf*-VII	*Gp*-D	*Gr*
Borne	47° 11′ 38″ N, 5° 27′ 09″ E	303	–	80	93	225
Brizotte	47° 12′ 17″ N, 5° 26′ 32″ E	314	–	–	174	225
Ecoulotte	47° 29′ 14″ N, 5° 34′ 12″ E	–	372	–	253	–
Morte	47° 26′ 48″ N, 5° 41′ 57″ E	256	–	–	208	237
Ouche	47° 17′ 55″ N, 5° 02′ 22″ E	–	226	22	229	231
Résie	47° 19′ 29″ N, 5° 32′ 23″ E	23	349	–	244	–
Romaine	47° 31′ 54″ N, 5° 53′ 04″ E	322	–	289	–	237
Vèze	47° 14′ 01″ N, 5° 34′ 38″ E	–	178	–	187	–
Vingeanne	47° 20′ 52″ N, 5° 27′ 09″ E	271	143	–	96	181

were kept at −20°C until processed for measurements, genetic identification and dissections.

Each amphipod was sexed and its fourth coxal plate was measured as a proxy for body size (Bollache et al. 2000). Because individuals with a fourth coxal plate height of <1 mm cannot be sexed and are never infected, we only used individuals larger than that size threshold (Lagrue et al. 2007, 2011). Amphipods have indeterminate growth, inducing a strong age–size correlation (Sutcliffe et al. 1981, Goos et al. 2014). All amphipods were then dissected and classified as infected or uninfected. Acanthocephalan parasites were morphologically identified and counted. Although mis-identifications of adult *Pomphorhynchus laevis* and *P. tereticollis* in their fish hosts are common and molecular data are needed to unambiguously separate these 2 parasite species, this is not the case for their larval stages (Špakulová et al. 2011). Cystacanths of *P. laevis* and *P. tereticollis* in their amphipod hosts are easily identifiable based on morphology; *P. laevis* cystacanths are small, spherical, smooth and light yellow, while *P. tereticollis* cystacanths are oblong, large, wrinkled and dark yellow (Perrot-Minnot 2004, Perrot-Minnot et al. 2019).

All amphipods were genetically assigned to their respective MOTU using the cytochrome *c* oxidase subunit I (COI as in Levy et al. 2002, Pfeiffer et al. 2004): *Gf*-I, *Gf*-III, *Gf*-VII or *Gp*-D (Lagrue et al. 2014). These MOTUs have divergences ranging between 3.8% when comparing *Gf*-I and *Gf*-III and 27% when comparing *Gf*-III and *Gp*-D (Lagrue et al. 2014). Contrastingly, and due to its invasive status, *Gr* populations are genetically homogeneous and all *Gr* sampled (N = 1336) are grouped in 1 MOTU (*Gr*; Moret et al. 2007). Amphipod DNA was extracted following Lagrue et al. (2014). Overall, 4632 amphipods were amplified for COI using LCO1490 and HCO2198 primers (Folmer et al. 1994). Each individual was then assigned to a MOTU (see Lagrue et al. 2014 for details).

Acanthocephalan infection levels (prevalence and abundance; see Bush et al. 1997 for terminology) were estimated for each MOTU in each population. Larval stages of 3 acanthocephalan species were found: *P. laevis*, *P. tereticollis* and *Polymorphus minutus*. The 2 *Pomphorhynchus* species use a variety of fish species, and *P. minutus* uses waterfowl as definitive hosts (Holmes & Bethel 1972, Kennedy 2006, Kaldonski et al. 2008b, Jacquin et al. 2014). All 3 species have been used in previous studies and have variable infection patterns and differential effects on hosts (Bauer et al. 2000, 2005, Cézilly et al. 2000, Bollache et al. 2001, 2002, Tain et al. 2006, 2007, Lagrue et al. 2007, Cornet et al. 2010). The prevalence and abundance of each parasite species were calculated for each amphipod MOTU and sampling site separately.

2.2. Statistical analyses

We compared parasite prevalences and abundances among MOTUs and for each parasite species separately using generalized mixed-effect models considering MOTU as a fixed effect variable and river (i.e. amphipod sampling site) as a random effect. Prevalences were assumed to follow a binomial distribution, whereas abundances were assumed to follow a Poisson distribution. To assess the relative importance of MOTU and sampling site in explaining the variance in prevalence and abundance, we compared estimated random effect variances of the 2 predictors in models where they are both treated as random effects. For each parasite species, we also compared parasite-induced mortality among MOTUs. If amphipods suffer from increased mortality with age due to the infection, we do not expect to observe old, and thus large, individuals carrying many parasites, as only uninfected individuals should survive to the larger sizes. In addition, because parasite infection is a stochastic process, we expect small, and thus younger, individuals to be less abundantly parasitized than their intermediate-sized conspecifics, because haphazard accumulation of parasites takes time. All else being equal, parasite abundance should therefore show a bell-shaped relationship to amphipod age/body size in populations where parasites induce mortality, whereas it should increase with age/size in populations where parasites do not affect host mortality. To test these predictions, we used a generalized mixed-effect model for a Poisson distribution considering parasite abundance as the response variable, the quadratic effect of amphipod body size (as a proxy of amphipod age), amphipod MOTU and their interaction as fixed-effect variables and the river where amphipods were collected as the random variable. A significant effect of the interaction between the quadratic effect of amphipod body size and MOTU indicates significant differences in parasite-induced mortality among MOTUs.

Among 5923 collected amphipods, we found 17 amphipods co-infected with a combination of 2 of the 3 parasite species. We excluded these observations from our analyses of parasite-induced mortality because in such cases, it cannot be determined which of the parasite species may induce host mortality,

and/or interspecific parasite co-infections may have cumulative or antagonistic effects on hosts. We also excluded observations from MOTUs in which fewer than 15 individuals in a river were infected by a focal parasite species. Under such low prevalences, it is difficult to estimate accurately the shape of the relationship between parasite abundance and host body size. Overall, we tested parasite-induced mortality in the following combinations of parasite species–host MOTU: *P. laevis*–(*Gf*-I, *Gr*), *P. tereticollis*–(*Gf*-I, *Gf*-III, *Gf*-VII, *Gp*-D, *Gr*) and *P. minutus*–(*Gf*-III, *Gr*). Finally, we compared amphipod mean body size among MOTUs using a linear mixed effect model using river as the random effect variable. Amphipod body size was standardized prior to statistical analyses. Mixed-effect models were analysed using the R packages 'lme4' (Bates et al. 2015) and 'multicomp' (Hothorn et al. 2008).

3. RESULTS

Overall, infection patterns and parasite-induced mortality differed widely among parasite species–host MOTU combinations, and significantly more than among sampling sites. Parasite prevalence and abundance varied significantly among parasite–MOTU combinations (Tables 2 & 3, Fig. 1). Although infection patterns differed slightly among sampling sites (Tables 2 & 3), prevalence and abundance varied more among MOTUs than sampling sites. This was indicated by the relatively low standard deviation of the random effect of river in our models compared to the estimated standard deviation of the MOTU effect when treated as a random variable (Table 4). Only *Polymorphus minutus* prevalence and abundance, which were generally low in all MOTUs and populations, tended to vary more among rivers than among MOTUs (Tables 3 & 4). *Gf*-I and *Gf*-III MOTUs showed higher parasite prevalence than the other 2 native MOTUs (*Gf*-VII and *Gp*-D) for all 3 parasite species (Fig. 1A, Table 2). Noticeably, *Gp*-D was never infected by *P. minutus* and *Gf*-VII never contained *Pomphorhynchus laevis* larvae, even though they co-occurred with amphipod MOTUs that were infected with *P. minutus* and *P. laevis*, respectively (Table 2, Fig. 1A). *Gf*-I and *Gf*-III also showed higher parasite abundances than *Gf*-VII and *Gp*-D (Table 3, Fig. 1B).

The general trends in abundance among MOTUs did not reflect parasite-induced mortality patterns. MOTUs differed in their mortality induced by *P. tereticollis*, as indicated by the significant effect of the interaction between MOTU and the quadratic effect of individual size on *P. tereticollis* abundance (likelihood ratio test: $\chi^2 = 31.782$, df = 8, $p < 0.001$). Specifically, individuals of *Gf*-III did not seem to suffer from increased mortality as a result of *P. tereticollis* infection, leading to a general increase in parasite abundance with body size/age (Fig. 2). In contrast, *Gf*-I, *Gf*-VII and *Gp*-D individuals showed a bell-shaped relationship between parasite abundance and host age, characteristic of parasite-induced mortality (Fig. 2). Different parasite species also affected their host differently. Although *Gf*-I individuals appeared to be influenced by mortality due to *P. tereticollis*, they did not seem affected by *P. laevis* infections (Fig. 2). On the other hand, although

Table 2. Parasite prevalences (proportion of infected individuals in %) among rivers and amphipod molecular operational taxonomic units (MOTUs) for the 3 acanthocephalan species separately: *Pomphorhynchus tereticollis*, *P. laevis* and *Polymorphus minutus*. (–) rivers where MOTUs were absent; 0 values indicate rivers where MOTUs were present but never infected with the focal parasite. MOTU abbreviations as in Table 1

River	MOTU				
	Gf-I	*Gf*-III	*Gf*-VII	*Gp*-D	*Gr*
P. tereticollis					
Borne	7.6	–	0	2.2	3.1
Brizotte	8.3	–	–	0	2.2
Ecoulotte	–	16.1	–	2.4	–
Morte	6.3	–	–	0	3.4
Ouche	–	8.4	0	0.9	0
Résie	17.4	16.9	–	1.2	–
Romaine	8.1	–	5.9	–	3.0
Vèze	–	61.2	–	4.3	–
Vingeanne	30.6	28.7	–	6.3	1.1
P. laevis					
Borne	0	–	0	0	9.3
Brizotte	0.3	–	–	0	8.0
Ecoulotte	–	0.3	–	2.4	–
Morte	0	–	–	0	16.0
Ouche	–	0	0	0	3.5
Résie	0	0.3	–	0.8	–
Romaine	1.6	–	0	–	10.1
Vèze	–	1.1	–	1.1	–
Vingeanne	7.4	4.9	–	0	15.5
P. minutus					
Borne	2.3	–	1.3	0	2.2
Brizotte	0.3	–	–	0	3.6
Ecoulotte	–	0.8	–	0	–
Morte	0	–	–	0	7.6
Ouche	–	0	0	0	0
Résie	0	4.0	–	0	–
Romaine	0.3	–	0.3	–	4.6
Vèze	–	3.4	–	0	–
Vingeanne	0.7	0	–	0	0.6

Table 3. Parasite abundances (mean number of parasites per host and [99% inter-quantile range of the distribution of the number of parasites per host]) among rivers and amphipod molecular operational taxonomic units (MOTUs) for the 3 acanthocephalan species separately: *Pomphorhynchus tereticollis*, *P. laevis* and *Polymorphus minutus*. (–) indicate rivers where MOTUs were absent; 0 values indicate rivers where MOTUs were present but never infected with the focal parasite. MOTU abbreviations as in Table 1

River	MOTU				
	Gf-I	*Gf*-III	*Gf*-VII	*Gp*-D	*Gr*
P. tereticollis					
Borne	0.07 [0, 1.49]	–	0 [0, 0]	0.02 [0, 1]	0.04 [0, 1]
Brizotte	0.11 [0, 2]	–	–	0 [0, 0]	0.02 [0, 1]
Ecoulotte	–	0.36 [0, 6.3]	–	0.03 [0, 1]	–
Morte	0.09 [0, 2]		–	0 [0, 0]	0.04 [0, 1]
Ouche	–	0.08 [0, 1.9]	0 [0, 0]	0.02 [0, 1]	0 [0, 0]
Résie	0.17 [0, 1]	0.24 [0, 3.3]	–	0.02 [0, 1]	–
Romaine	0.10 [0, 2.4]	–	0.06 [0, 1]	–	0.05 [0, 1]
Vèze	–	2.14 [0, 9.6]	–	0.05 [0, 2]	–
Vingeanne	0.70 [0, 6]	0.54 [0, 6.2]	–	0.16 [0, 5]	0.03 [0, 1.1]
Total	0.20 [0, 5]	0.55 [0, 8.66]	0.05 [0, 1]	0.03 [0, 1]	0.03 [0, 1]
P. laevis					
Borne	0 [0, 0]	–	0 [0, 0]	0 [0, 0]	0.11 [0, 2]
Brizotte	0.003 [0, 0.84]	–	–	0 [0, 0]	0.11 [0, 2.9]
Ecoulotte	–	0.003 [0, 0.8]	–	0.03 [0, 1]	–
Morte	0 [0, 0]	–	–	0 [0, 0]	0.22 [0, 3]
Ouche	–	0 [0, 0]	0 [0, 0]	0 [0, 0]	0.03 [0, 1]
Résie	0 [0, 0]	0.003 [0, 0.8]	–	0.01 [0, 0.78]	–
Romaine	0.02 [0, 1]	–	0 [0, 0]	–	0.09 [0, 1.82]
Vèze	–	0.03 [0, 2.15]	–	0.01 [0, 1]	–
Vingeanne	0.13 [0, 3]	0.06 [0, 1.29]	–	0 [0, 0]	0.06 [0, 1.1]
Total	0.03 [0, 2]	0.01 [0, 1]	0 [0, 0]	0.01 [0, 1]	0.11 [0, 2]
P. minutus					
Borne	0.02 [0, 1]	–	0.02 [0, 1.21]	0 [0, 0]	0.02 [0, 1]
Brizotte	0.003 [0, 0.84]	–	–	0 [0, 0]	0.04 [0, 1]
Ecoulotte	–	0.008 [0, 1]	–	0 [0, 0]	–
Morte	0 [0, 0]	–	–	0 [0, 0]	0.09 [0, 2]
Ouche	–	0 [0, 0]	0 [0, 0]	0 [0, 0]	0 [0, 0]
Résie	0 [0, 0]	0.06 [0, 2.5]	–	0 [0, 0]	–
Romaine	0.003 [0, 0.84]	–	0.003 [0, 0.86]	–	0.05 [0, 1]
Vèze	–	0.03 [0, 1]	–	0 [0, 0]	–
Vingeanne	0.007 [0, 0.65]	0 [0, 0]	–	0 [0, 0]	0.005 [0, 0.1]
Total	0.01 [0, 1]	0.02 [0, 1]	0.01 [0, 16]	0 [0, 0]	0.03 [0, 1]

P. tereticollis was not associated with increased mortality in *Gf*-III, *P. minutus* infections appeared to induce mortality in this MOTU (Fig. 2). The invasive *Gr* generally showed higher prevalences and abundances of *P. laevis* and *P. minutus* than native amphipod MOTUs (Fig. 1). Infection levels of *P. tereticollis* in *Gr* were more variable compared to these observed in sympatric native MOTUs. Prevalences of *P. tereticollis* were similar in *Gr*, *Gf*-VII and *Gp*-D, and abundances were similar in *Gr*, *Gf*-I and *Gf*-III. Interestingly, while *Gr* clearly suffered from parasite-induced mortality when infected with *P. minutus* and *P. tereticollis*, *P. laevis* did not seem to induce mortality in this MOTU and simply accumulated in *Gr* individuals (Fig. 3). Finally, native MOTUs differed significantly in mean body size (likelihood ratio test: $\chi^2 = 1463.5$, df = 3, $p < 0.001$), with *Gf*-I and *Gf*-III amphipods being smaller than *Gf*-VII amphipods, which themselves were smaller than *Gp*-D individuals (Table 5).

4. DISCUSSION

Although *Gp* and *Gf* have long been considered as distinct species, despite unreliable diagnostic morphological features, recent molecular evidence shows that they are a species complex characterized by cryptic diversity (Karaman & Pinkster 1977, Westram et al. 2011a, Lagrue et al. 2014, Weiss et al.

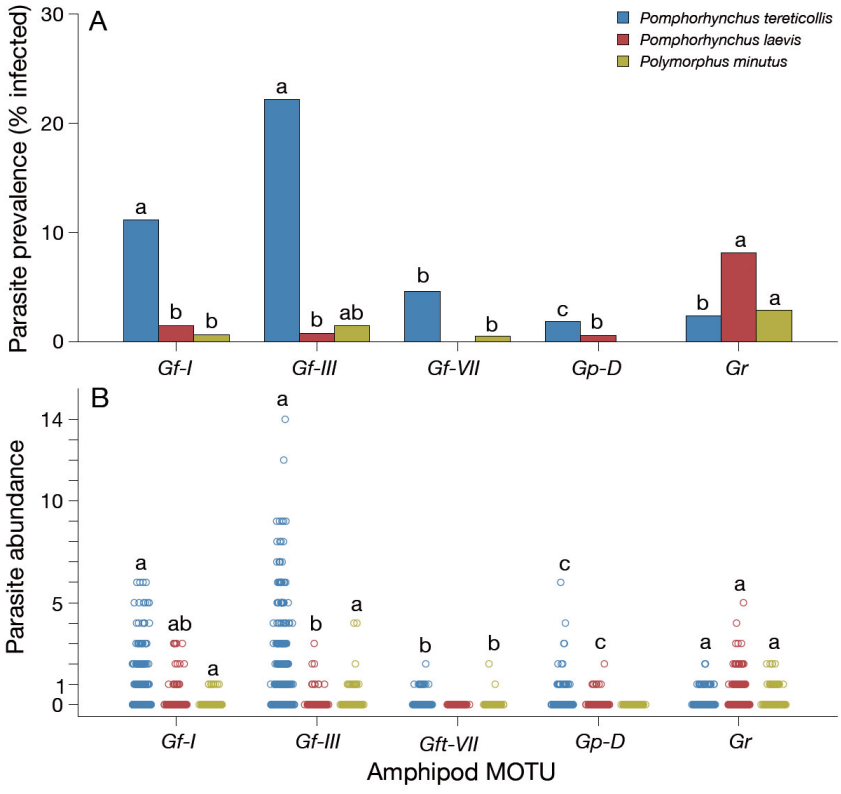

Fig. 1. Overall (all sampling sites combined) (A) parasite prevalence (proportion of infected individuals) and (B) parasite abundance (raw data on a log scale) among amphipod molecular operational taxonomic units (MOTUs) for the 3 acanthocephalan species separately; *Pomphorhynchus tereticollis*, *P. laevis* and *Polymorphus minutus*. Prevalences or abundances assigned different letters are significantly different among MOTUs at the 0.05 level. Differences were calculated for each parasite species separately. We added a slight horizontal jitter on abundance data points for representation purposes. MOTU abbreviations as in Table 1

2014). Such cryptic diversity can have profound implications when it comes to assessing parasite infection levels, parasite-induced mortality and other life history traits for which amphipods are traditional study models (Galipaud et al. 2015a,b, 2017). Here, we found that infection patterns of 3 acanthocephalan species were significantly different among MOTUs; prevalence and abundance were actually more strongly influenced by host MOTU than by amphipod sampling site. Furthermore, prevalence, abundance and parasite-induced mortality vary widely among host MOTU–parasite species combinations. Cryptic diversity has significant and potentially large implications in apparent infection patterns. For example, some MOTUs are never infected by some acanthocephalan species, even though they co-occur with amphipod MOTUs that are infected. Such patterns are only evident in light of cryptic diversity. It thus begs the question: have previous studies missed an important factor by involuntarily overlooking cryptic diversity? What are the potential consequences on previously documented patterns? Below, we discuss these potential effects on studies performed in the same sampling areas as the present.

4.1. Comparison of infection patterns between native and invasive amphipods

Invasive host species are often assumed to be less susceptible to native parasites and/or less affected by the pathological effects of infection than native hosts when exposed to local parasites (Torchin et al. 2003, Prenter et al. 2004, Roche et al. 2010, Lagrue 2017). However, such trends were not clear from parasite prevalences or abundances documented here and elsewhere (Galipaud et al. 2017). A recurrent research area at the University of Bourgogne has been to compare the effects of local parasites on native and invasive amphipods using *Gp* and *Gr* as study models. Early on, it was shown that acanthocephalans displayed different infection patterns and unequally affected native and invasive host species, mainly to the advantage of the invader (Bauer et al. 2000, 2005, Kaldonski et al. 2007, 2008b). For example, Cornet et al. (2010) found that pathological effects of *Pomphorhynchus laevis* affected the native *Gp* more than the invasive *Gr*,

Table 4. Relative dispersion in prevalence and abundance values among molecular operational taxonomic units (MOTUs) or sampled rivers (site). Dispersions were measured using estimated standard deviations of the random effects of sampling site and MOTU predictor variables

Parasite	Dispersion (SD_{random})			
	Prevalence		Abundance	
	Site	MOTU	Site	MOTU
Pomphorhynchus tereticollis	0.888	1.017	1.086	1.094
Pomphorhynchus laevis	0.460	1.048	0.562	1.044
Polymorphus minutus	1.020	0.766	1.120	0.742

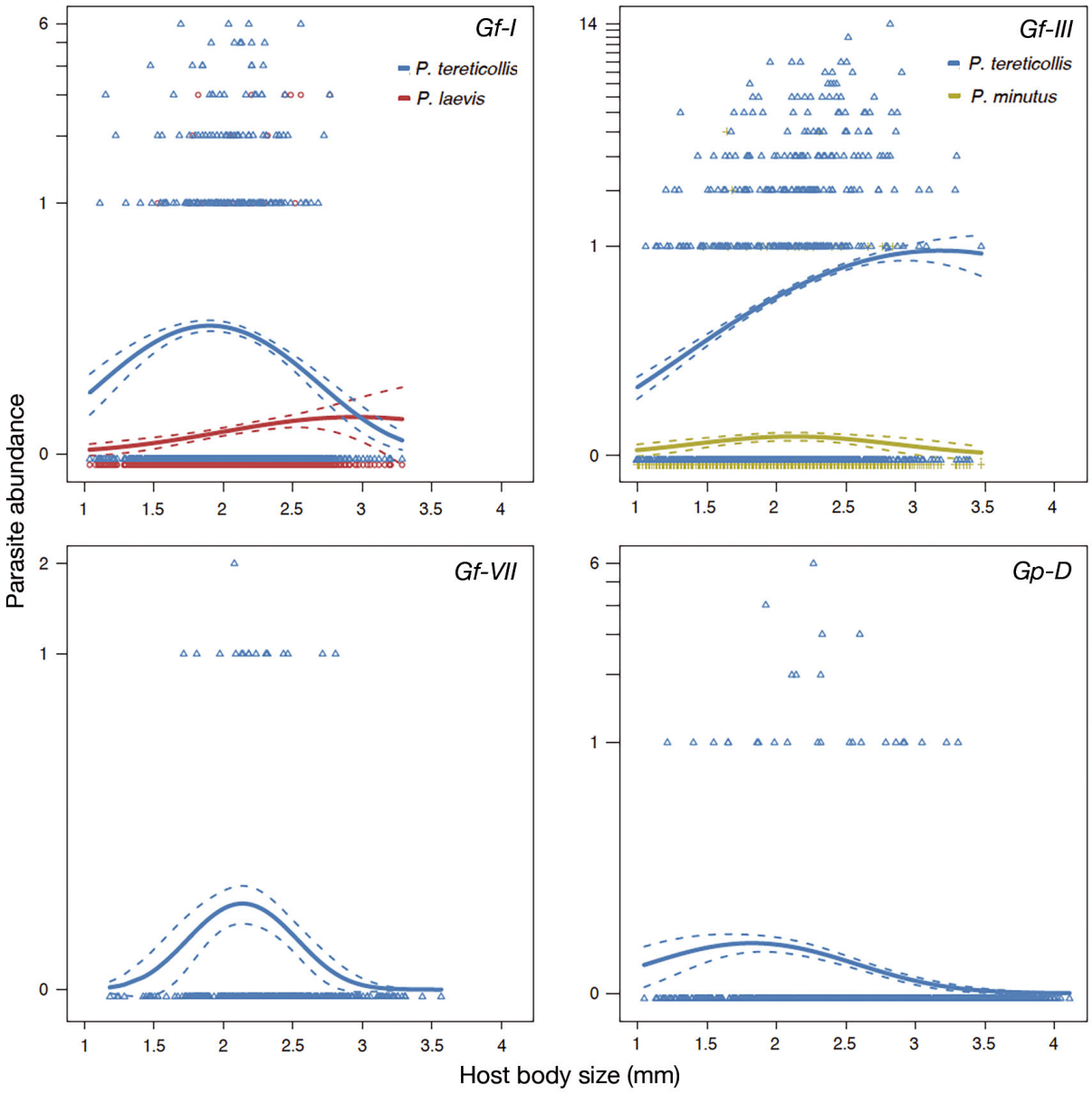

Fig. 2. Parasite-induced mortality among amphipod molecular operational taxonomic units (MOTUs) and parasite species (*Pomphorhynchus tereticollis*: blue triangles; *P. laevis*: red circles; *Polymorphus minutus*: yellow crosses). Parasite abundance in hosts are plotted as a function of host body size (fourth coxal plate height, in mm), taken as a proxy for host age. Predicted curves (solid lines) and their standard error (dotted lines) represent the estimated quadratic effect of host body size on parasite abundance. A bell-shaped relationship indicates parasite-induced mortality. We plotted the *y*-axis on a log scale, rescaled body size to initial values and slightly shifted down data points corresponding to an abundance of 0 for representation purposes. MOTU abbreviations as in Table 1

potentially providing the invader with a competitive advantage through lower parasite-induced mortality. While it is indeed the case for *P. laevis* in some native MOTUs, we have shown here that acanthocephalan parasites have diverse, species-specific effects on different *Gp*/*Gf* MOTUs' infection patterns and parasite-induced mortality. For instance, *P. laevis* did not appear to induce mortality in the native amphipod *Gf*-I.

Overall, the situation is more complicated than previously thought due to the co-occurrence of cryptic MOTUs. These studies often selectively sampled amphipod individuals infected with a particular species of acanthocephalan from a host population containing several MOTUs and may have thus compared MOTUs with completely different vulnerabilities to infection. Kaldonski et al. (2007) used

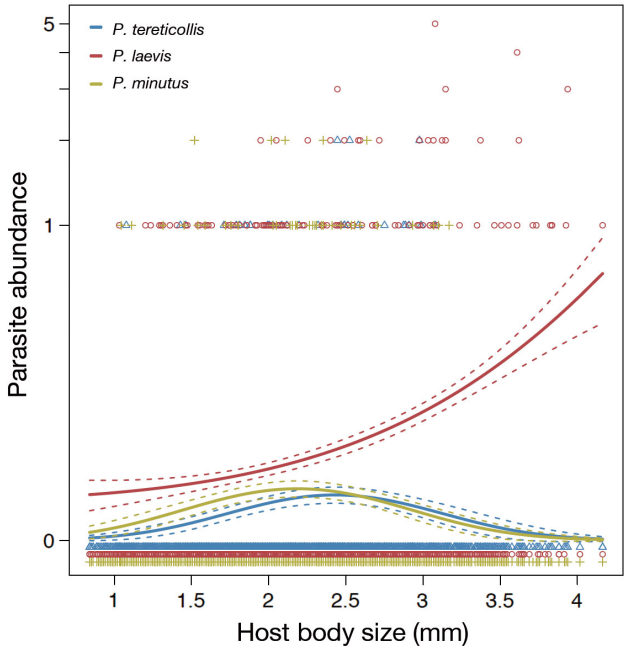

Fig. 3. Parasite-induced mortality in *Gammarus roeseli*. Details as in Fig. 2

amphipods infected by *P. laevis* and *Polymorphus minutus* to compare their effects on their 'common host' from the River Ouche, where 3 native MOTUs (*Gf*-III, *Gf*-VII and *Gp*-D) with parasite-specific infection patterns are present. In light of our data, these hosts are actually mostly parasite-specific. Patterns of invasive host advantage may thus differ significantly according to which native MOTUs the invader is competing with, varying from situations where invasive hosts are significantly less affected than native hosts by local parasites to situations where the reverse is true.

In a wider context, it is becoming increasingly obvious that invasive crustacean hosts are not always colonizing new areas on their own but may carry their own acanthocephalan parasites with them (Hohenadler et al. 2018). Whether these invasive parasites will spill over in the population of native hosts or the native parasites spill back on the newly available hosts is highly context dependent but will strongly affect local host–parasite dynamics and infection levels, and potentially host abundance (van Riel et al. 2003, Emde et al. 2012, Lagrue 2017). Since some of our amphipod populations also contained the invasive *Gr*, there is a possibility that some of the parasites we found were also invasive, which may have affected the documented infection levels among host MOTUs and/or the frequency of each MOTU in the population. Generally, cryptic diversity should also be considered in a biological invasion context, both at the host and parasite levels.

Table 5. Pairwise comparisons of body size differences among molecular operational taxonomic units (MOTUs). MOTU abbreviations as in Table 1

Comparisons	Mean size difference	SE	p
Gf-I vs. *Gf*-III	0.024	0.025	0.75
Gf-I vs. *Gf*-VII	−0.33	0.027	<0.0001
Gf-I vs. *Gp*-D	−0.56	0.020	<0.0001
Gf-III vs. *Gf*-VII	−0.35	0.037	<0.0001
Gf-III vs. *Gp*-D	−0.58	0.017	<0.0001

4.2. Variations in parasite effects on hosts

Historically, acanthocephalan infection patterns and other important life-history traits of amphipods have been assessed at the population level, a unit that was assumed to be homogeneous. One of the earliest studies on acanthocephalan infection in amphipods at the University of Bourgogne collected amphipods infected with *P. laevis* or *P. minutus* in the River Ouche, assuming that that all individuals were *Gp*, to compare the specific effects of each parasite on their host (Cézilly et al. 2000). However, we now know that this population contains 3 native MOTUs (*Gf*-III *Gf*-VII and *Gp*-D) that display widely different infection patterns and species-specific parasite-induced mortality. The consequences of cryptic diversity on the conclusions drawn by these studies are unclear but potentially large.

Most subsequent comparisons were made among host populations or between parasite species found in the same host population (Outreman et al. 2002, 2007, Cornet et al. 2007, 2009a,b, Lagrue et al. 2007, 2013, Médoc et al. 2011). The majority of studies conducted at the host population level show that acanthocephalan parasites induce host mortality in their amphipod hosts and produce the characteristic bell-shaped curve of parasite abundance across amphipod size/age (Lagrue et al. 2007, 2013, Outreman et al. 2007). Some studies found parasite species-specific effects on *Gr* and *Gp*, taxonomic species regarded as homogeneous at the time (Tain et al. 2006, 2007). Others found that parasite richness (number of acanthocephalan species) and prevalence were variable among amphipod populations but assumed that resistance to infection was population specific (i.e. local adaptation) and unrelated to parasite species;

some populations were hypothesized to be more resistant than others or that parasites were simply more abundant in some rivers than others (Cornet et al. 2009b).

Acanthocephalan parasites, especially *P. laevis* and *P. minutus*, manipulate amphipod behaviour, resulting in higher transmission to the final host and a negative effect of infection on their intermediate host's survival (e.g. Bakker et al. 1997, Lagrue et al. 2007). Studies comparing diverse parasite and host species combinations to assess differences or commonalities in host manipulation and infection patterns are common (Ponton et al. 2006, Perrot-Minnot et al. 2014). Some studies also detected variations in parasite effects among populations of *P. laevis* and *Gp* (Franceschi et al. 2008, 2010). Altogether, these studies hinted at sampling site effects to explain variations in infection patterns; hosts and parasites were suggested to have coevolved different manipulation abilities or resistance. Franceschi et al. (2010) attempted to test general patterns of local adaption in parasite infectivity and host manipulation abilities using 6 amphipod populations that were thought to be a single species, *Gp*, at the time. In hindsight, these populations contained 8 different cryptic MOTUs (*Gf*-I, *Gf*-II, *Gf*-III, *Gf*-VI, *Gp*-A, *Gp*-B, *Gp*-C and *Gp*-D). We show here that at least 3 of these MOTUs (*Gf*-I, *Gf*-III and *Gp*-D) display variable susceptibility to *P. laevis* and are differently affected by acanthocephalan parasites. Similarly, results presented by Cornet et al. (2009b) may have been strongly influenced by cryptic diversity and the MOTU composition of each sampled population. Indeed, that study used amphipods from several sampling sites (i.e. the Rivers Ouche, Vingeanne, Suzon and Meuzin) that have since been documented as containing at least 6 MOTUs and potentially more, as not all rivers have been tested for cryptic diversity. A number of studies at the University of Bourgogne were conducted on amphipods from the Rivers Ouche and Vingeanne (e.g. Cézilly et al. 2000, Tain et al. 2006, 2007, Lagrue et al. 2007, Kaldonski et al. 2008a,b, 2009, Cornet et al. 2009c). Present data show that these 2 rivers alone each contain 3 native MOTUs of the *Gp*/*Gf* complex and the invasive *Gr*; these MOTUs display widely different susceptibility to infection and species-specific parasite-induced mortality (Figs. 1–3). We showed that infection patterns are more variable among MOTUs than among sampling sites, rendering any test of local host–parasite adaptation among amphipod populations made up of cryptic MOTUs largely ineffective. In light of the present results, the potential effects of cryptic diversity on documented patterns of variation in parasite infection are thus likely to be critically important.

4.3. Inter-MOTU differences in body size and parasite-induced mortality patterns

MOTUs not only differed in infection and abundance patterns, but also in average body size. Failure to recognize that several MOTUs of various sizes coexist in sympatry could strongly affect estimations of parasite-induced mortality in previous studies. For example, the mortality of *Gf*-III MOTUs is unaffected by *P. tereticollis* infection, while *Gp*-D is clearly affected by *P. tereticollis*-induced mortality. Incidentally, *Gf*-III amphipods are also much smaller than *Gp*-D individuals. In populations containing both *Gf*-III and *Gp*-D MOTUs, estimations of parasite-induced mortality could have been biased when performed unaware of such diversity in amphipod hosts. Specifically, the presence of large uninfected *Gp*-D individuals suggests a strong parasite-induced mortality at the population level, therefore hiding the fact that large *Gf*-III individuals are in fact heavily parasitized. These MOTUs have been found to coexist in the River Vingeanne, the amphipod population of which has repeatedly been sampled in previous parasitology studies (e.g. Cornet et al. 2007, 2009b, Médoc et al. 2011). Oblivious of cryptic diversity and MOTU-specific infection patterns, these studies likely misinterpreted patterns of parasite-induced mortality.

4.4. When amphipods of different MOTUs are used in experimental parasite infection

Experimental cross-infection among populations of hosts and parasites is a common tool used to test local host–parasite co-adaptations or variations in infection patterns among amphipod populations (Franceschi et al. 2008, Dianne et al. 2010, 2011). In such studies, to ensure that they were free of parasites before experimental infections, amphipods were often sampled from populations deprived of the focal acanthocephalan species (Cornet et al. 2009b, Perrot-Minnot et al. 2012, 2014, Dianne et al. 2014). Furthermore, a large majority of these studies used amphipods from a specific site (i.e. the River Suzon) that was recently revealed to contain 3 cryptic MOTUs, while sourcing parasites from sites with further cryptic diversity and the occurrence of different MOTUs (Franceschi et al. 2008, 2010, Cornet et al. 2009a, Dianne et al. 2010,

2011, 2014, Lagrue et al. 2014). These studies often compared the effects of acanthocephalan parasites on amphipod host behaviour and mortality between naturally infected individuals from one sampling site and experimentally infected amphipods from another population. Again, sampling sites regularly included the Rivers Ouche and Suzon (Franceschi et al. 2008, 2010, Dianne et al. 2010). However, cryptic MOTU compositions of these 2 populations are very different (Lagrue et al. 2014); the Suzon contains a majority of *Gp*-A individuals with a minority of *Gf*-I and *Gf*-II amphipods (Lagrue et al. 2014), while the Ouche population is mainly composed of *Gf*-III and *Gp*-D individuals with a minority of *Gf*-VII (Table 1). Infection patterns and parasite-induced mortality, and thus most likely behavioural host manipulation, vary widely among these MOTUs (Figs. 1 & 2; Galipaud et al. 2017). In one instance, natural and experimental infections actually used amphipod hosts from the same population (Cornet et al. 2009c). Uninfected adult amphipods were selected for experimental infections and exposed to acanthocephalan eggs. However, these amphipods again came from the Ouche, a population made up of 3 divergent MOTUs with highly contrasting infection patterns (Figs. 1 & 2; Lagrue et al. 2014, Galipaud et al. 2017). By selectively choosing naturally infected individuals and comparing them to naturally uninfected individuals experimentally exposed to infection, the studies may have simply compared infection patterns between host MOTUs without realizing it. The conclusions drawn by these studies need to be reassessed in light of amphipod cryptic diversity and its complex effects on infection patterns.

4.5. Cryptic diversity: from hosts to parasites

Recently, cryptic diversity has been discovered in multiple helminth groups (Pérez-Ponce de León & Poulin 2018). Several studies have indeed revealed previously unknown levels of cryptic diversity in various acanthocephalan species, including *P. minutus* and several species of the genus *Pomphorhynchus* (Perrot-Minnot et al. 2018, Zittel et al. 2018, Reier et al. 2019). These results indicate that cryptic MOTUs in parasite species and their respective degree of host specificity may be underestimated and strongly influence host–parasite dynamics and observed infection patterns. Although we did not collect data on parasite genetics for our study, both published and unpublished data from members of the same laboratory strongly suggest that there is no cryptic diversity within or among sampling sites for the 2 fish parasites *P. laevis* and *P. tereticollis* (Vardić Smrzlić et al. 2015, Perrot-Minnot et al. 2018, R. Wattier & M. J. Perrot-Minnot unpubl. data). In contrast, information for *P. minutus* in eastern France is non-existent, so there is a possibility that some level of cryptic diversity may occur in our study systems and may have influenced our results. Adding to this complexity, cryptic lineages of *P. minutus* are highly host specific (Zittel et al. 2018). In the context of our study, it is thus possible that the *P. minutus* infection patterns we observed are influenced by the combination of cryptic diversity in both host and parasite.

Acanthocephalans are commonly considered to have low specificity towards their intermediate hosts (Dudiňák & Šnábel 2001). However, although *P. minutus* seems to use a wide range of amphipod species as intermediate hosts, it was noticed very early on that infection success of parasite eggs ingested by amphipods was very high provided that the species of amphipod host was the same as that used by the previous generation of parasites (Hynes & Nicholas 1958). Although there was no concept of cryptic diversity at the time, it was clear that host specificity was high among 'strains' of *P. minutus*, which may have been linked to cryptic diversity in the parasite as recently discovered (Zittel et al. 2018). Similar patterns have been observed in *P. laevis* (O'Mahony et al. 2004). Such apparent host specificity among taxonomic and cryptic species of parasites combined with the existence of sympatric host MOTUs are thus likely to strongly influence natural infection patterns in amphipod–acanthocephalan associations. Overall, although cryptic diversity in parasites may have had limited effects on the present results, studies documenting infection patterns in natural populations and testing host–parasite dynamics in laboratory settings should assess cryptic diversity in both hosts and their parasites to ensure that all potential factors are accounted for (Pérez-Ponce de León & Nadler 2010, Pinacho-Pinacho et al. 2018).

4.6. Concluding remarks

Cryptic diversity and its complex effects should be considered when assessing infection patterns in amphipod populations, as divergent MOTUs can display highly contrasting and parasite species-specific patterns of susceptibility to infection and parasite-induced mortality. Overall, there is mounting evidence for widespread cryptic diversity in amphipod taxa and highly variable sensitivities to contaminants

and parasites among cryptic MOTUs (Müller 2000, Sutherland et al. 2010, Westram et al. 2011b, Cothran et al. 2013a,b, Lagrue et al. 2014, Weiss et al. 2014, Galipaud et al. 2017). Previously, researchers, including ourselves, were simply and genuinely unaware of cryptic diversity in natural populations because of the lack of tools to detect it. Cryptic diversity is, by definition, not obvious and needs to be actively sought out. Erroneous interpretation of data, albeit genuine, are therefore likely to continue to happen in fields that are not using molecular and genetic techniques. It is becoming evident that cryptic diversity can have profound impacts on widely studied ecological patterns, and willingly ignoring this fact could hurt the credibility of further work on these patterns.

Our results show that infection patterns (prevalence, abundance and parasite-induced mortality) were the result of complex and unpredictable interactions between amphipod host MOTU and parasite species. Given the constantly decreasing costs and increasing ease of use of molecular techniques and reagents, cryptic diversity should be routinely tested for in amphipod taxa. Given that many aquatic organisms have limited dispersal abilities, we suggest that cryptic diversity be examined in other aquatic hosts to ensure that any biological or ecological patterns are not influenced by this clearly pervasive factor. Moreover, cryptic diversity in parasites will add another layer of complexity to host–parasite association patterns, and thus represents another factor to assess in diversity studies (Zittel et al. 2018). Some recent studies have started on that path and have opened the way towards routine application of such techniques in research using biological models with high degrees of cryptic diversity (Bauer & Rigaud 2015, Labaude et al. 2015, 2017, Zittel et al. 2018).

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